The BRCA-1 binding protein BRAP2 is a novel, negative regulator of nuclear import of viral proteins, dependent on phosphorylation flanking the nuclear localization signal

Alex J. Fulcher,* Daniela M. Roth,* Shadma Fatima,*,† Gualtiero Alvisi,*,1 and David A. Jans*,†,2
*Nuclear Signaling Laboratory, Department of Biochemistry and Molecular Biology, and †Australian Research Council Centre of Excellence for Biotechnology and Development, Monash University, Clayton, Victoria, Australia

ABSTRACT This study describes for the first time the ability of the novel BRCA1-binding protein 2 (BRAP2) to inhibit the nuclear import of specific viral proteins dependent on phosphorylation. Ectopic expression of BRAP2 in transfected African green monkey kidney COS-7 cells was found to significantly reduce nuclear localization signal (NLS)-dependent nuclear accumulation of either simian virus SV40 large-tumor antigen (T-ag) or human cytomegalovirus DNA polymerase processivity factor ppUL44; this was also observed in HL-60 human promyelocytic leukaemia cells on induction of BRAP2 expression by vitamin D3 treatment. BRAP2 inhibition of nuclear accumulation was dependent on phosphorylation sites flanking the respective NLSs, where substitution of the cyclin-dependent kinase site T124 of T-ag with Ala or Asp prevented or enhanced BRAP2 inhibition of nuclear import, respectively. Substitution of T427 within the NLS of ppUL44 gave similar results, whereas no effect of BRAP2 was observed on nuclear targeting of other viral proteins, such as herpes simplex virus-1 pUL30, which lacks a phosphorylation site near its NLS, and the human immunodeficiency virus-1 Tat protein. Pulldowns/AlphaScreen assays indicated direct, high-affinity binding of BRAP2(442–592) to T-ag(111–135), strictly dependent on negative charge at T124 and the NLS. All results are consistent with BRAP2 being a novel, phosphorylation-regulated negative regulator of nuclear import, with potential as an antiviral agent.—Fulcher, A. J., Roth, D. M., Fatima, S., Alvisi, G., and Jans, D. A. The BRCA-1 binding protein BRAP2 is a novel, negative regulator of nuclear import of viral proteins, dependent on phosphorylation flanking the nuclear localization signal. J. Cell Biol. 24, 1454–1466 (2010). www.fasebj.org

Key Words: SV40 large-tumor antigen · T-ag · cytomegalovirus processivity factor ppUL44 · cytoplasmic retention factor · nuclear transport regulation · p53 tumor suppressor

Nuclear protein transport is a highly regulated process that requires members of the importin (IMP) superfamily of proteins that mediate movement into and out of the nucleus through the nuclear envelope-localized nuclear pore complexes (NPCs) (1, 2). Proteins targeted to the nucleus by IMPs generally carry a basic nuclear localization signal (NLS), the best understood pathway being that where the IMPα/β heterodimer recognizes the NLS through IMPα, and IMPβ mediates passage through the NPC and release within the nucleus compartment on interaction with the GTP-binding protein Ran (2).

Phosphorylation/dephosphorylation is the best-understood mechanism of regulation of nuclear transport, mediated by many different kinases/phosphatases (2–4), with phosphorylation in the vicinity of NLSs a key mechanism by which NLS function can be regulated (2–4). This is mediated by various mechanisms (4–15), including modulation of NLS accessibility either positively (5, 9, 12, 13, 15, 16) or negatively (10, 11, 13), and specific retention in the cytoplasm, as has been observed for the glucocorticoid receptor (17) and the transcription factor NF-κB (7, 14). A well-characterized example is that of the simian virus SV40 large-tumor antigen (T-ag), where phosphorylation at serines 111/112 (protein kinase CK2 site) and serine 120 (double-stranded DNA dependent protein kinase site) close to the NLS (PKKRRK)132 enhances IMPα/β recognition ~100-fold and thereby increases nuclear import ~50-fold (9, 15). In contrast, phosphorylation of threonine 124 by cyclin-dependent kinase (cdk) inhibits nuclear import, believed to be through a cytoplasmic retention mechanism (16). Another viral protein that has recently been described as possessing a phosphorylation-regulated NLS similar to that of T-ag is the cytomegalovirus (CMV) ppUL44, where CK2-site

1 Current address: Department of Molecular Virology, University of Heidelberg, Heidelberg, Germany.
2 Correspondence: Nuclear Signaling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Wellington Rd., Clayton, Victoria 3800, Australia. E-mail: david.jans@med.monash.edu.au doi: 10.1096/fj.09-136564

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phosphorylation of serine 413 and possibly serine 415 enhances IMPα/β binding to the NLS (PNTKKQ<sub>431</sub>), thereby increasing nuclear import (5), while phosphorylation at threonine 427 appears to inhibit nuclear import (unpublished results). The molecular basis of inhibition of T-ag/ppUL44 nuclear import through cytoplasmic retention has not been investigated.

BRCA1 binding protein 2 (BRAP2) was first identified as a cytoplasmic localized protein able to interact with the NLS of the tumor suppressor and breast cancer antigen BRCA1, as well as those from T-ag and mitosis (18). More recently, it has been reported as playing a role in cell differentiation by retaining the universal cdk inhibitor p21<sup>cip1</sup> in the cytoplasm during monocyte differentiation (19), implying its potential as an inhibitor of nuclear entry of specific nuclear acting factors. In the present study, we examine for the first time the functional consequences of interaction with BRAP2 on nuclear transport of T-ag and ppUL44, two viral proteins that are imported into the nucleus by the IMPα/β heterodimer, and their nuclear import modulated by specific phosphorylation sites close to the respective NLSs. The results indicate that BRAP2 can act to inhibit NLS-dependent nuclear protein import dependent on specific phosphorylation near the NLS, implicating BRAP2 as a novel negative regulator of nuclear import (NRNI) of viral proteins and possibly other molecules in specific phosphorylation-dependent fashion.

MATERIALS AND METHODS

**T-ag expression plasmid construction using Gateway technology**

All mammalian expression constructs encoding GFP-BRAP2, DsRed2- and GFP-T-ag, -UL44, -UL30, and -Tat fusion proteins used in this study were generated using the Gateway system (Invitrogen, Carlsbad, CA, USA). Primers for each of the gene products that included either the attB1 (5'-3') or attB2 (3'-5') recombination sites were used to amplify the gene from their respective templates; BRAP2 (18), T-ag (20), UL44 (5), or Tat (21). PCR fragments were introduced into plasmid vector pDONR207 (Invitrogen) via the BP recombination reaction, according to the manufacturer’s recommendations, to generate the entry clones pDONR207-BRAP2-2, pDONR207-BRAP2-3 (343–592), pDONR207-BRAP2-4 (442–592), pDONR207-BRAP2-5 (2,584–3,283), pDONR207-BRAP2-6 (2,584–3,283), pDONR207-BRAP2-7 (110–135), pDONR207-BRAP2-8 (110–135) D<sup>124</sup>, pDONR207-BRAP2-9 (110–135) A<sup>124</sup>, pDONR207-BRAP2-10 (410–433), pDONR207-BRAP2-11 (410–433) D<sup>127</sup>, and pDONR207-BRAP2-12 (1,013). These entry clones, together with the previously described pDONR207-UL30 (1,114–1,136) (22), were then used to perform LR recombination reactions with Gateway system-compatible expression vectors to produce pDsRed2 (23) and pEPI-GFP (24) constructs, according to the manufacturer’s recommendations, in order to express DsRed2- and GFP-tagged fusion proteins in mammalian cell systems, while LR recombination reactions into Gateway-compatible pDEST15 (Invitrogen) and pGFPattC (25) bacteria expression vectors to express GST- and H<sub>i</sub>GH<sub>4</sub>F-GFP fusion proteins, respectively, enabled protein purification. The pDsRed2-UL44(2–433) expression construct has been previously described (26). BRAP 343–592 coding sequence was also inserted into pDsRed2-C1 (Clontech, Mountain View, CA, USA) between the HindIII and BamHI restriction endonuclease sites, fused in frame C-terminal to DsRed2. The mammalian expression plasmids encoding GFP-T-ag(111–135) or the T<sup>128</sup> mutant were made using restriction/ligation, as previously described (27).

**Cell culture and transfection**

COS-7 cells were maintained grown in DMEM containing 10% FCS in a humidified incubator at 37°C. They were seeded into 6- and 12-well plates 1 d prior to transfection, performed using Lipofectamine 2000 (Invitrogen), according to the recommendations of the manufacturer, for use in confocal laser scanning microscopy (CLSM) experiments, as previously described (26). HL-60 cells were maintained in RPMI 1640 medium containing 10% FCS; where appropriate, BRAP2 expression was induced by treatment with 100 nM vitamin D<sub>3</sub> for 96 h (19), prior to experimentation. HL-60 cells with or without prior vitamin D<sub>3</sub> treatment were transfected by electroporation (200 V, 800 mF) using a Bio-Rad Gene Pulser II (Bio-Rad, Hercules, CA, USA).

**CLSM and image analysis**

Endogenously expressed T-ag and p53 were visualized in COS-7 cells following fixation with 4% (w/v) paraformaldehyde, by incubating with either anti-T-ag (sc-20800; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-p53 (Cell Signaling Technology, Danvers, MA, USA) rabbit polyclonal antibodies and anti-hnRNP A1 (Sigma, St. Louis, MO, USA) mouse monoclonal, followed by Alexa 568 labeled goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR, USA) or Alexa 568 labeled goat anti-mouse secondary antibody (Molecular Probes). Samples were mounted on coverslips in 4% propyl-gallate made in PBS/glycerol (90% w/v) and imaged using Olympus Fluoview 1000 CLSM (Olympus, Tokyo, Japan), with a Nikon ×60 oil-immersion lens (Nikon, Tokyo, Japan). Subcellular localization of GFP and DsRed2 fusion proteins in living COS-7 cells was visualized 21 h after transfection using the Fluoview CLSM, equipped with differential interference contrast (DIC) and Nikon ×60 water-immersion lens in combination with a heated stage (Nikon). Image analysis to quantify relative subcellular localization was performed as previously described (5, 9–11, 15, 16, 22–24, 26, 28–33), whereby the nuclear (F<sub>n</sub>) and cytoplasmic (F<sub>c</sub>) fluorescence ratios (F<sub>n/c</sub> and F<sub>c/n</sub>, respectively) were determined from single-cell measurements using the ImageJ 1.41o public domain software (National Institutes of Health (NIH), Bethesda, MD, USA), subsequent to the subtraction of background fluorescence due to autofluorescence. In some cases, the relative volume of the nuclear and cytoplasmic compartment was taken into account in order to enable an estimate of the absolute levels of cellular protein in each (33). This _in situ_ quantitative analysis avoids potential problems associated with the redistribution of proteins on subcellular fractionation _etc._ (34, 35), and is representative of cell populations, rather than an individual cell, through the averaging of results for multiple live cells for each construct/treatment.

**Immunoprecipitation/Western blot analysis**

GFP-fusion proteins were immunoprecipitated using an anti-GFP (mouse monoclonal; Roche, Basel, Switzerland) antibody, according to the recommendations of the manufacturer. Briefly, cells (10-cm dishes/80% confluent) were harvested 48 h post-transfection and lysed in 300 μl of lysis
buffer (150 mM NaCl, 1% Triton X-100, and 50 mM Tris-HCl, pH 7.5), and complete protease inhibitors (Roche), and incubated on ice for 30 min. Cell debris was removed by centrifuging at 14,000 rpm for 20 min at 4°C, and the supernatant was incubated overnight at 4°C with 2.8 μg of anti-GFP antibody. Thirty microfilters of Protein A/G Plus-agarose (Santa Cruz Biotechnology) was prewashed with lysis buffer, added to the lysate/antibody mix, and incubated for 4 h at 4°C. The beads were pelleted by centrifugation at 10,000 rpm for 1 min, before washing 3 times for 15 min in 1 ml of lysis buffer, centrifuging and discarding the supernatant each time. The final wash was 1 ml 50 mM Tris-HCl (pH 7.5).

Proteins were eluted from the beads using 50 μl of SDS lysis buffer (0.2% bromophenol blue, 10% glycerol, 200 mM DTT, 2% SDS, and 100 mM Tris; pH 8.3), 25 μl of the eluate was subjected to polyacrylamide gel electrophoresis (8% gel), and the separated proteins were then transferred to a nitrocellulose filter (26). The membrane was blocked in buffer A (5% skim milk powder (w/v) and 1× PBS) overnight at 4°C and washed 3 times with buffer B (0.05% Tween and 1× PBS).

Detection of GFP-BRAP2 fusion proteins or endogenous BRAP2 was performed by incubating membranes with anti-BRAP2 (1:1000; Sigma) rabbit primary antibody and HRP-coupled goat anti-rabbit secondary antibody (1:10,000; Chemicon, Temecula, CA, USA). Detection of SV40 T-ag and αβ tubulin was performed analogously, except that anti-SV40 T-ag (v900, 1:750; Santa Cruz Biotechnology) or anti-αβ tubulin (1:1000; Cell Signaling Technology)-specific antibodies were used. Lamin B1 was detected using an anti-Lamin B1 (1:750; Zymed, Carlsbad, CA, USA) mouse primary antibody, and HRP-coupled goat anti-mouse secondary antibody (1:10,000; Chemicon). A rabbit polyclonal anti-GFP antibody (1:2500; Abcam, Cambridge, UK) was used to detect GFP-fusion proteins as per the manufacturer’s instructions, followed by incubation with HRP-coupled goat anti-rabbit secondary antibody as above. The immunoblots were incubated with ECL plus reagent (Amersham Biosciences, Piscataway, NJ, USA), according to the recommendations of the manufacturer, and chemiluminescence was captured on a photosensitive film.

**Densitometric analysis**

Band intensity captured on film was measured on densitometric scans using the NIH ImageJ 1.41o public domain software, with the membrane background subtracted. Band intensity was then calculated relative to a loading control such as Lamin B1, in the case of nuclear extracts, or to GFP-fusion proteins expression level in the case of the pulldown experiments. Results were expressed relative to the appropriate control for each experiment.

**FACS and nuclear extraction**

COS-7 cells expressing either GFP-BRAP2(2–592) or GFP alone were sorted using an Influx 488/561 dual laser FACS sorter into GFP-negative and GFP-expressing cells. To prepare nuclear extracts, sorted cells were pelleted and resuspended in nuclei isolation buffer (NIB; 320 mM sucrose and 0.5% Triton X-100) made in 1× intracellular buffer (IB; 110 mM KCl, 5 mM NaHCO₃, 5 mM MgCl₂, 1 mM EGTA, 0.1 mM CaCl₂, 20 mM HEPES, and 1 mM dithiothreitol, pH 7.4) to a final volume of 10⁷ cells/ml, and left on ice for 5 min. The nuclei were harvested by centrifugation at 2000 g for 5 min at 4°C, and then the supernatant (cytoplasmic extract) was discarded. Nuclei were washed twice with nuclei wash buffer (NIB without Triton X-100), centrifuged for 5 min at 4°C, and then resuspended to a final volume of 2 × 10⁷ nuclei/ml using 1× PBS (1% CHAPS) for electrophoresis/Western blot analysis.

**Protein expression and purification**

All His₆-GFP-T-ag fusion proteins were expressed and purified as described previously (36). GST-BRAP2 was expressed in *Escherichia coli* host strain BL21(DE3). Cultures grown at 37°C, OD₆₅₀ of 0.6 were induced with 1 mM IPTG for 3 h. Bacterial pellets were resuspended in PBS and 1 mM DTT (PBS buffer) containing 500 μg/ml lysozyme and Complete EDTA-free protease inhibitors (Roche), then lysed by sonication, and cell debris was removed by centrifugation. The supernatant was incubated with GST-bead slurry for 2 h at 4°C, washed in PBSD, and eluted in 50 mM Tris (pH 8.0) and 200 mM NaCl containing 20 μM reduced glutathione. Glutathione was removed *via* dialysis; the protein was transferred to PBS buffer and then concentrated prior to biotinylation using the Sulfo-NHS-Biotin reagent (Pierce, Rockford, IL, USA) as previously described (36). Free biotin was removed using a PD-10 column (Amersham Biosciences), and the protein was concentrated by centrifugation using a 3 × 10⁴ MW column (Millipore, Bedford, MA, USA). Protein estimation was performed using Bio-Rad reagent (24) with BSA as a standard.

**AlphaScreen assay**

Analysis of direct binding of His₆-GFP-T-ag derivatives to GST-BRAP2(442–592) or -IMPs was performed using an established AlphaScreen assay (Perkin Elmer, Wellesley, MA, USA) (36). Briefly, 30 nM of His₆-GFP-T-ag protein was bound to Ni²⁺-chelate acceptor beads and incubated with increasing concentrations of biotinylated GST-tagged BRAP2(442–592) or IMPS or GST alone bound to streptavidin-coated donor beads. In the case of binding of the IMPα/β complex, biotinylated IMPα was first predimerized to nonbiotinylated IMPβ at 13.6 μM for 15 min at RT in 1× IB (15, 16). Binding interactions were then detected using a FusionA (Perkin Elmer) plate reader (36), and sigmoidal curves were fitted using SigmaPlot software (Systat, San Jose, CA, USA) to calculate the apparent dissociation constant ($K_d$).

**RESULTS**

**BRAP2 is a cytoplasmic protein able to inhibit the nuclear localization of viral proteins**

To assess BRAP2’s potential role as an NRNI, we generated a GFP-tagged BRAP2 expression construct together with truncated derivatives thereof (Fig. 1); the N-terminal BRAP2(2–376) construct includes the E3 ubiquitin ligase domain but not the coiled-coil domain, whereas the coiled-coil domain, but not the ubiquitin ligase domain, is present in the C-terminal BRAP2(343–592) construct. The constructs were transiently expressed in COS-7 cells, and their subcellular localization is analyzed by CLSM. Strictly cytoplasmic localization was observed for all three BRAP-GFP fusion proteins, in contrast to GFP alone, which showed a diffuse distribution throughout the cell (Fig. 1).

We had previously shown that T-ag nuclear localization is regulated by the cdk site adjacent to the NLS, whereby phosphorylation reduces nuclear accumulation, not by influencing recognition of the NLS by
IMPα/β (ref. 9; see Supplemental Fig. 1), but through an apparent cytoplasmic retention mechanism (10). As a first step to test whether T-ag nuclear localization is affected by BRAP2, we overexpressed GFP-BRAP2(2–592) or GFP alone in COS-7 cells, which constitutively express T-ag, and subsequently fixed and immunostained the cells for T-ag (Fig. 2A). Results for CLSM analysis to determine the levels of T-ag nuclear accumulation in cells expressing BRAP2 were compared to those for nontransfected cells (see Material and Methods). The average nuclear fluorescence $F_n$ of T-ag in the presence of GFP-BRAP2(2–592) was found to be significantly reduced (~20%; $P=0.0018$), accompanied by a significant increase (~2-fold; $P=0.0007$) in the average cytoplasmic fluorescence $F_c$ compared to in its absence (Fig. 2B, left and center panels). Calculation of the nuclear to cytoplasmic ratio ($F_n/c$), as previously described (9, 10, 12, 15, 16, 22, 30, 37), indicated a significant reduction (~60%; $P=0.0037$) in nuclear accumulation in the presence of GFP-BRAP2(2–592) compared to in its absence (Fig. 2B, right panel); taking into account the relative volume of cellular nuclear and cytoplasmic compartments (32, 33), overexpressing BRAP2 resulted in an overall estimated redistribution from nucleus to cytoplasm of 17% of cellular T-ag compared to in its absence. Notably, the reduced $F_n/c$ values for T-ag in cells overexpressing GFP-BRAP were not attributable to bleedthrough from the green to the red channel (see Supplemental Fig. 2), while overexpression of GFP alone did not affect T-ag nuclear accumulation, highlighting the specificity of the effects to BRAP2.

**Figure 1.** Schematic and subcellular localization of BRAP2 constructs used. COS-7 cells were transfected to express the indicated GFP-BRAP2 fusion constructs or GFP alone, and imaged live 21 h post-transfection using CLSM. Right panels: representative images and nuclear to cytoplasmic ratio ($F_n/c$, determined as described in Materials and Methods) for each construct ($n>23$).

**Figure 2.** BRAP2 can act as an NRNI for T-ag. A) CLSM images of COS-7 cells transfected to express GFP-BRAP2(2–592), followed by fixation 16 h post-transfection and immunostaining using a specific anti-T-ag antibody (middle) and an Alexa568 labeled secondary antibody. B) Image analysis of CLSM files represented in A, performed as per Fig. 1: average $F_n$ (left), $F_c$ (middle), and $F_n/c$ ratio (right) ($n>62$); $F_n$ and $F_c$ values are expressed as percentages relative to T-ag in the absence of exogenous BRAP2 expression. Significant differences in the absence or presence of BRAP2 are indicated by $P$ values. C) Cells were transfected to express either GFP-BRAP2(2–592) or GFP alone and FACS sorted 21 h later, prior to preparation of nuclear extracts as described in Materials and Methods. Left panel: samples were electrophoresed and subjected to Western blot analysis for T-ag, Lamin B1, and αβ tubulin as indicated, using specific antibodies. Right panel: results for densitometric analysis; values are calculated relative to COS-7 cells not expressing GFP fusion proteins (see Materials and Methods). D) COS-7 cells were transfected to express GFP or GFP-BRAP2 constructs as indicated; cell lysates were prepared 30 h post-transfection and subjected to pulldown using anti-GFP antibody as per Materials and Methods. BRAP2 (top) and T-ag (bottom) were detected using specific antibodies.
To confirm the results, COS-7 cells were transfected to express GFP-BRAP2(2–592) or GFP alone and FACS sorted to separate GFP-expressing and nonexpressing cells, followed by the preparation of nuclear extracts. Western/densitometric analysis revealed a ~50% reduction in the levels of nuclear T-ag in COS-7 cells expressing GFP-BRAP2(2–592), compared to cells not expressing GFP or expressing GFP alone (Fig. 2C), consistent with the results in Fig. 2B. Immunoprecipitation using COS-7 cells expressing GFP-BRAP2 constructs (aa 2–592, 2–376, 343–592) or GFP alone and anti-GFP antibody showed that T-ag could be pulled down in a complex with BRAP2 from cells expressing GFP-BRAP2(2–592) or GFP-BRAP2(343–592), but not from those expressing GFP-BRAP2(2–376) or GFP alone (Fig. 2D). The clear implication was that the BRAP2 C-terminus is responsible for complexation with T-ag that results in reduced nuclear accumulation.

We have recently characterized the nuclear import pathway of HCMV ppUL44, the CMV polymerase DNA processivity factor; intriguingly, the NLS of ppUL44 strongly resembles that of T-ag in being recognized by IMPα/β, and containing several phosphorylation sites immediately upstream of the NLS that modulate its function (5, 22). We decided to test the effect of ectopic expression GFP-BRAP2 on the subcellular localization of DsRed2-T-ag(110–135) and -UL44 (2–433) fusion proteins, containing the respective NLS and flanking phosphorylation sites, which regulate their nuclear import (Fig. 3A). Although the extent of cytoplasmic localization of GFP-BRAP2 did not appear to be affected by coexpression of either DsRed2-T-ag(110–135) or DsRed2-UL44 (2–433) (data not shown), the average \( F_n \) of DsRed2-T-ag(110–135) was shown to be significantly reduced (>20%; \( P<0.009 \)) in the presence of either GFP-BRAP2(2–592) or GFP-BRAP2(343–592), compared to in the absence of GFP-BRAP2; in parallel, the average \( F_n \) significantly increased (~2-fold; \( P<0.0003 \)), relative to in the absence of GFP-BRAP2, as well as in the presence of GFP-BRAP2(2–376) (Fig. 3B, left and center panels). Calculation of the \( F_{n/c} \) ratio showed that nuclear accumulation of DsRed2-T-ag(110–135) (Fig. 3B, right panel) was significantly \( (P<0.0001) \) reduced in the presence of either GFP-BRAP2(2–592) or GFP-BRAP2(343–592) \( (F_{n/c} \) values of 29 and 39, respectively), but not GFP-BRAP2(2–376) \( (F_{n/c}=68; \) Fig. 2D), indicating that the BRAP2 C-terminus is sufficient for the inhibition of T-ag nuclear accumulation. We also performed experiments using DsRed2-BRAP2(343–592) or DsRed2 alone and GFP-T-ag (110–135) i.e., with the GFP/DsRed2 tags exchanged between T-ag and BRAP2, a significant \( (P<0.0001) \) reduction in nuclear accumulation of GFP-T-ag was observed in cells coexpressing DsRed2-BRAP2 but not DsRed2 alone (see Supplemental Fig. 3), the clear implication being that the inhibitory effect of BRAP2 overexpression on T-ag nuclear accumulation is not due to the various fusion tags used.

In similar fashion to the results for T-ag, the average \( F_n \) of DsRed2-UL44 (2–433) was found to be signifi-

and \( n>24 \); \( F_n \) and \( F_{n/c} \) values are expressed as percentages relative to DsRed2-T-ag(110–135) or DsRed2-UL44 (2–433) fusion proteins either alone or coexpressed with GFP-BRAP2 fusion proteins 21 h post-transfection. Channel images: left, GFP (green); right, DsRed2 (red). B, C) Image analysis of cells represented in A: average \( F_n \) (left), \( F_{n/c} \) (middle), and \( F_{n/c} \) ratio (right) \( (n>27 \)

Figure 3. BRAP2 can act as an NРNI for the viral proteins SV40 T-ag and CMV UL44. A) CLSM images of COS-7 cells transfected to express DsRed2-T-ag(110–135) or DsRed2-UL44 (2–433) fusion proteins either alone or coexpressed with GFP-BRAP2 fusion proteins 21 h post-transfection. Channel images: left, GFP (green); right, DsRed2 (red). B, C) Image analysis of cells represented in A: average \( F_n \) (left), \( F_{n/c} \) (middle), and \( F_{n/c} \) ratio (right) \( (n>27 \)

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significantly reduced (~20%; \( P = 0.035 \)) in the presence of either GFP-BRAP2(2–592) or GFP-BRAP2(343–592) (Fig 3A, C) and was accompanied by a significant (\( P < 0.0003 \)) increase (2-fold) in the average \( F_{nc} \), relative to that in the absence of BRAP2 (Fig. 3A, C), or in the presence of GFP-BRAP2(2–376). The calculated \( F_{nc} \) values further supported this idea, whereby the extent of nuclear accumulation of DsRed2-T-ag(110–135) was significantly (\( P < 0.0001 \)) reduced in the presence of either GFP-BRAP2(2–592) or GFP-BRAP2(343–592) (Fig 3A, C).

**TABLE 1. Summary of the activity of BRAP2 as an NRNI with respect to various viral proteins and the control molecule DsRed2**

<table>
<thead>
<tr>
<th>DsRed2 fusion protein</th>
<th>+GFP-BRAP2 (2–592)</th>
<th>+GFP-BRAP2 (343–592)</th>
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<tbody>
<tr>
<td>T-ag (110–135)</td>
<td>40.3 ± 1.5 (7)</td>
<td>47.2 ± 3.2 (6)</td>
</tr>
<tr>
<td>T-ag (110–135) ( D^{124} )</td>
<td>58.9 ± 5.4 (5)</td>
<td>62.3 ± 5.7 (4)</td>
</tr>
<tr>
<td>T-ag (110–135) ( A^{124} )</td>
<td>115.3 ± 9.1 (4)</td>
<td>90.5 ± 9.8 (3)</td>
</tr>
<tr>
<td>UL44 (410–433)</td>
<td>40.4 ± 2.5 (5)</td>
<td>35.6 ± 5.4 (4)</td>
</tr>
<tr>
<td>UL44 (410–433) ( D^{427} )</td>
<td>57.3 ± 24.1 (2)</td>
<td>44.9 ± 27.3 (2)</td>
</tr>
<tr>
<td>UL44 (410–433) ( A^{427} )</td>
<td>101.1 ± 34.5 (2)</td>
<td>88.1 ± 11.3 (2)</td>
</tr>
<tr>
<td>UL44 (2–433)</td>
<td>64.2 ± 4.9 (6)</td>
<td>57.2 ± 6.9 (6)</td>
</tr>
<tr>
<td>UL30 (1114–1136)</td>
<td>91.3 ± 14 (2)</td>
<td>98.5 ± 10 (1)*</td>
</tr>
<tr>
<td>HIV-1 Tat (2–101)</td>
<td>104.6 ± 16.6 (3)</td>
<td>78.8 ± 16.4 (3)</td>
</tr>
<tr>
<td>DsRed2 (alone)</td>
<td>98.2 ± 1.9 (2)</td>
<td>99.5 ± 1.29 (2)</td>
</tr>
</tbody>
</table>

Summary of data from cotransfection experiments (\( n \) shown in parentheses), such as those shown in Figs. 3, 4, and 6. Results are for the \( F_{nc} \) (mean ± se, \( n \) in parentheses) in % relative to the value in the absence of GFP-BRAP2 expression. A significant reduction is seen in the presence of both GFP-BRAP2 proteins in all cases, with the exception of the control molecules DsRed2-Tat (2–101) and DsRed2 alone, and the alanine-substituted T-ag (110–135) and UL44 (410–433) derivatives. *se from variation in individual measurements for a single experiment (\( n > 45 \).

**Figure 4.** Inhibition of nuclear accumulation of T-ag by BRAP2 is dependent on the \( T^{124} \) phosphorylation site flanking the NLS. A) CLSM images for COS-7 cells transfected to express DsRed2-T-ag(110–135) fusion protein or the \( D^{124} \) and \( A^{124} \) derivatives in the absence or presence of coexpressed GFP-BRAP2 fusion proteins 21 h post-transfection. Channel images: left, GFP (green); right, DsRed2 (red). B) Image analysis, performed as per Fig. 1(\( n > 19 \)). \( P \) values are indicated for significant differences in \( F_{nc} \) in the absence (−) or presence of BRAP2 expression, and between cells expressing the DsRed2-T-ag(110–135) \( T^{124} \) or \( D^{124} \) derivatives. C) Lysates of COS-7 cells transfected to express either GFP, GFP-T-ag(111–135:T\( D^{128} \)) (NLS mut), or the indicated GFP-T-ag(110–135) derivatives were immunoprecipitated using an anti-GFP antibody as described in Materials and Methods prior to detection by Western blot analysis of BRAP2 (top) or the GFP fusion proteins themselves (bottom) using specific antibodies. D) Densitometric analysis to estimate levels of protein in C. Value for BRAP2 above background was calculated relative to that of GFP/GFP fusion protein; values are expressed in terms of GFP alone (left) or as percentage of GFP-T-ag(110–135) WT relative to GFP (right).
or GFP-BRAP2(343–592) ($F_{n/c}$ of 34 and 40, respectively), but not GFP-BRAP2(2–376) ($F_{n/c}$=63), compared to that in the absence of BRAP2 ($F_{n/c}$=71). Coexpression with GFP-BRAP2 did not alter the nucleo-cytoplasmic distribution of the control molecule DsRed2 alone (data not shown; $F_{n/c}$~1 in all cases), or the extent of nuclear or nucleolar (indicated by the nucleolar to cytoplasmic ratio, $F_{nu/c}$) accumulation of either the herpes simplex virus type 1 (HSV-1) UL30(1114–1136)-DsRed2 fusion protein ($F_{n/c}$ and $F_{nu/c}$~40 and 60, respectively, in all cases; Fig. 3D, E) or human immunodeficiency virus type 1 (HIV-1) Tat(2–101)-DsRed2 fusion protein ($F_{n/c}$ and $F_{nu/c}$~6 and 30, respectively, in all cases; data summarized in Table 1). UL30 contains a bipartite NLS recognized by IMPα/β (22), whereas Tat is normally imported into the nucleus by IMPβ1 with no requirement for IMPα (38); the NLS is not modulated in activity by flanking phosphorylation sites in either case. The clear implication was that BRAP2’s ability to inhibit nuclear accumulation does not apply generally to viral proteins (see summary in Table 1), but rather is specific to proteins bearing a phosphorylation site close to the basic core of an IMPα/β-recognized NLS (see below).

Regulation of T-ag and ppUL44 nuclear import by BRAP2 is modulated by phosphorylation

Phosphorylation by cdk or aspartic acid substitution of threonine 124 reduces NLS-dependent nuclear accumulation of T-ag (10). HCMV ppUL44 is similar (5), in that aspartic acid substitution of threonine 427, a putative PKC site, inhibits nuclear import (unpublished data; see Fig. 6). In the case of T-ag, recognition by IMPα/β is not significantly affected by negative charge at the phosphorylation sites (ref. 9; see Supplemental Fig. 1), implying that the effects are not due to impaired IMP binding. To test whether specific phosphorylation may modulate BRAP2 inhibition of T-ag and ppUL44 nuclear import, DsRed2-T-ag(110–135) fusion derivatives generated with either aspartic acid or alanine in place of threonine 124 were coexpressed with various GFP-BRAP2 constructs, and results were compared to those for wild type (WT). As previously described (10), nuclear accumulation of the D$^{124}$ derivative ($F_{n/c}$=45) was significantly reduced ($P<0.05$) compared to the WT and the A$^{124}$ derivative ($F_{n/c}$=60; Fig. 4A, B), which is attributable to the action of endogenous BRAP2 in COS7 cells (see below; Fig. 4C). Nuclear accumulation of the D$^{124}$ derivative was further reduced significantly ($P<0.05$) on coexpression with either GFP-BRAP2(2–592) or GFP-BRAP2(343–592), in comparable fashion to the WT construct (Fig. 4B). In contrast, no significant effect of BRAP2 overexpression was observed on nuclear accumulation of the A$^{124}$ derivative compared to in its absence ($F_{n/c}$~60). The results imply clearly that negative charge at T$^{124}$ facilitates BRAP2 binding to the T-ag NLS, while alanine at the site oblates the inhibitory effect of BRAP2 on T-ag nuclear localization; that BRAP2’s inhibition of WT T-ag nuclear accumulation is not complete in living cells is largely attributable to the fact that <=100% of T-ag is phosphorylated at T$^{124}$ (10, 39).

To confirm that BRAP2 interacts directly with T-ag, we performed an AlphaScreen binding assay (36), using bacterially expressed His$_6$GFP-T-ag(110–135) (WT) and mutant derivatives thereof and GST-BRAP2(442–592), a truncated form of BRAP2 that was able to be expressed to sufficient levels in bacteria to enable purification. GST-BRAP2(442–592) was found to bind GFP-T-ag(110–135: D$^{124}$) to a much higher extent (>2-fold; Fig. 5A) than

![Figure 5](image)

Figure 5. BRAP2 C-terminus binds to T-ag(110–135) with high affinity dependent on negative charge at position 124. A) GFP-T-ag(110–135) WT and mutants as indicated were incubated with increasing concentrations of biotinylated GST-BRAP2(442–592), and an AlphaScreen assay was performed (see Material and Methods). Sigmoidal curves were fitted using Sigmaplot software to determine $B_{max}$ and $K_d$ values; results are expressed as a percentage relative to binding for GFP-T-ag(110–135:D$^{124}$). B) CLSM images of COS-7 cells transfected to express DsRed2-T-ag(110–135) WT in the absence (right panels) or presence of coexpressed GFP-BRAP2(343–592) or GFP-BRAP2(442–592) (left panels). C) Quantitative analysis of levels of nuclear accumulation in COS-7 cells expressing DsRed2-T-ag without or with GFP-BRAP2. Results represent mean ± se $F_{n/c}$ (n=40). $P$ values denote significant differences.
either GFP-T-ag(110–135) WT or GFP-T-ag(110–135; A124). GST-BRAP2(442–592) bound to GFP-T-ag(110–135; A124) with high affinity (\(K_d = 0.7 \pm 0.1\) nM, \(n = 3\); Fig. 5A); in addition to binding to a markedly lower extent, GFP-T-ag(110–135; A124) bound GST-BRAP2(442–592) with a \(3\)-fold lower affinity (\(K_d = 1.9 \pm 0.2\) nM). Only very low binding was observed for the GFP-T-ag fusion proteins to GST alone or of GST-BRAP2(442–592) to GFP alone, underlining the specificity of the binding interactions. Clearly, BRAP2(442–592) bound directly to T-ag(110–135), strongly dependent on negative charge at position 124, consistent with the idea (see above) that phosphorylation of T-ag at T124 is necessary for BRAP2 binding in transfected cells. That BRAP2(442–592) is able to inhibit nuclear import was confirmed by cotransfection experiments (Fig. 5B); nuclear accumulation of DsRed2-T-ag(110–135) (\(F_{n/c} = 45\)) was found to be significantly reduced (\(F_{n/c} = 15; P < 0.0001\)) in the presence of BRAP2(442–592), to an extent comparable to the effect of GFP-BRAP2(343–592) (\(F_{n/c} = 18\); Fig. 5C).

Similar effects to the results for T-ag and the T124 phosphorylation site were observed in transfected cells for ppUL44 with respect to the T427 phosphorylation site using DsRed2-UL44(410–433) fusion protein derivatives (Fig. 6A, B). The D427 mutant showed significantly reduced (\(P < 0.0001\)) nuclear accumulation compared to the WT (T427) and A427-substituted derivatives (\(F_{n/c} = 25\) compared to \(\sim 63\) and \(\sim 60\), respectively), again attributable to the action of endogenous BRAP2. GFP-BRAP2(2–592) coexpression significantly reduced nuclear accumulation of the D427 and WT derivatives, but not the A427 derivative (\(F_{n/c} = 25\), \(\sim 15\) and \(\sim 55\), respectively; Fig. 6B), with the same trend seen when GFP-BRAP2(343–592) was coexpressed. That nuclear accumulation of the D427 derivative seemed to be reduced to a greater extent than that of WT is attributable to the fact that the stoichiometry of phosphorylation at T427 is \(< 1.0\). The clear implication is that BRAP2 reduced nuclear accumulation of UL44 dependent on phosphorylation at T427, in similar fashion to the dependence of T-ag on phosphorylation at the T124 site.

To confirm that the effects observed were due to BRAP2 binding, COS-7 cells were transfected to express GFP-T-ag(110–135; WT, A124, or D124), with GFP alone as negative control, and anti-GFP antibody used to immunoprecipitate the proteins, followed by Western blot analysis.

Figure 6. BRAP2 inhibits the nuclear accumulation of UL44 dependent on the T427 phosphorylation site within the NLS. A) CLSM images of COS-7 cells transfected to express the DsRed2-UL44 (410–433) fusion protein or D427 or A427 derivatives in the absence or presence of coexpressed GFP-BRAP2 fusion proteins 21 h post-transfection. Channel images: left, GFP (green); right, DsRed2 (red). B) Image analysis, performed as per Fig. 1 (\(n > 24\)). \(P\) values are indicated for significant differences in \(F_{n/c}\) between cells in the absence (–) or presence of BRAP2 coexpression, and between cells expressing DsRed2-UL44 (410–433) T427 and D427 derivatives.
to assess the extent of association of endogenous BRAP2 to the GFP-T-ag(110–135) (Fig. 4C, D). As expected, BRAP2 was detected in pull downs with the T-ag(110–135) proteins; significantly, T-ag(110–135: D124) pulled down markedly higher amounts of endogenous BRAP2 protein than the GFP-T-ag(110–135:A124) or GFP-T-ag(110–135) WT. Densitometric analysis correcting for the extent of GFP/GFP-T-ag expression indicated that BRAP2 bound GFP-T-ag(110–135) WT almost 2-fold that of GFP alone, with the A124 derivative showing BRAP2 binding only 1.5-fold above GFP. In contrast, GFP-T-ag(110–135: D124) showed BRAP2 binding >4-fold that of GFP alone, almost 3-fold higher than that of GFP-T-ag(110–135) A124 (Fig. 4D). Clearly, association of BRAP2 with T-ag was strongly dependent on negative charge at T124, consistent with the direct binding data (Fig. 5A); to assess the NLS dependence of binding, comparable experiments were performed using an NLS mutant derivative of T-ag. As indicated in Fig. 4C, D, GFP-T-ag(111–135: T128) bound 75% less BRAP2 compared to GFP-T-ag(110–135) WT, the clear implication being that a functional NLS is required for BRAP2 recognition of T-ag, consistent with previous findings (18).

**BRAP2 up-regulation reduces nuclear accumulation of T-ag**

COS-7 cells express very low levels of endogenous BRAP2, making them ideal for ectopic expression studies as shown here. To confirm that the results relate to a system where BRAP2 expression is modulated by a physiological stimulus, the HL-60 promyelocytic leukemia cell system was used, whereby BRAP2 expression is strongly up-regulated on treatment of the cells with vitamin D3 (see Fig. 7C) (19). Accordingly, HL-60 cells without or with vitamin D3 treatment were transfected to express GFP-T-ag(111–135) or GFP alone and analyzed by CLSM (Fig. 7A). As in the case of COS-7 cells, the average Fn of GFP-T-ag(111–135) was significantly reduced (~20%; P=0.0006) in vitamin D3-treated HL-60 cells (high levels of BRAP2) when compared to untreated cells (low levels of BRAP2), accompanied by an almost 3-fold increase in average Fc (P<0.0001) (Fig. 7B, left and center panels). Determination of the Fn/c (Fig. 7B, right panel) indicated a significant (P<0.0001) reduction in nuclear accumulation of GFP-T-ag(111–135) in HL-60 cells expressing high

**Figure 7.** Induction of endogenous BRAP2 significantly reduces nuclear accumulation of GFP-T-ag(111–135). A) CLSM images are shown for HL-60 cells treated without or with vitamin D3 (VitD) to induce BRAP2 expression (19) transfected to express GFP-T-ag(111–135) or GFP alone 30 h post-transfection. Left, DIC images; right, GFP channel. B) Image analysis of cells represented in A: average Fn (left), Fc (middle), and Fn/c ratio (right) (n>15); Fn and Fc values are expressed as percentages relative to either GFP-T-ag(111–135) or GFP alone in the absence of VitD induction. P values denote significant differences in Fn/c for GFP-T-ag(111–135) expressed in cells without or with BRAP2 induction by VitD. C) Western blot analysis showing the expression of BRAP2 in HL-60 cells treated without or with VitD.
levels of BRAP2, compared to untreated cells expressing low levels ($F_{n/c}$ values of 24 and 7, respectively), consistent with the above results; this corresponds to an estimated overall redistribution from nucleus to cytoplasm of $\sim 18\%$ of GFP-T-ag on induction of BRAP2 expression by vitamin D3 compared to its absence. Notably, localization of the control GFP alone was not altered (Fig. 7B), underlining the specificity of the effects. Thus, an increase in endogenous BRAP2 expression also results in inhibition of nuclear accumulation of T-ag.

**BRAP2 can act as an NRNI for cellular proteins**

To test whether BRAP2 may be able to act as an NRNI for proteins other than viral gene products, COS-7 cells were transfected to express GFP-BRAP2(2–592) and fixed 16 h post-transfection, prior to immunostaining for the p53 tumor suppressor protein (Fig. 8A), which is normally imported by the IMPα/β heterodimer (40) or hnRNP A1 (Fig. 8A), which is transported into the nucleus by transportin (41–43), as a control. Quantitative analysis showed that p53 nuclear accumulation was significantly reduced ($\sim 60\%; P<0.0001$) in the presence of BRAP2 overexpression, accompanied by a close to 2-fold increase in $F_c$, equating to an overall $\sim 2.5$ fold reduction in $F_{n/c}$ (Fig. 8B), compared to its absence; this corresponds to an estimated overall redistribution from nucleus to cytoplasm of $\sim 23\%$ of p53 on BRAP2 overexpression compared to its absence. In contrast to p53, hnRNP A1 nuclear accumulation was not altered in the presence of BRAP2 overexpression (Fig. 8B). Thus, BRAP2 is able to inhibit the nuclear import of cellular proteins other than viral gene products, but this appears likely to be specific to proteins imported into the nucleus by IMPα/β (19).

Intriguingly, p53 contains a cdc2 phosphorylation site N-terminal to its NLS (SPQPKKKPL332) (44, 45), thus closely resembling the combination of cdc2 site and NLS in T-ag (10) and PK-C site and NLS in CMV UL44 (5) (see Fig. 9B), while p21Cip1, whose nuclear import has been reported to be inhibited by BRAP2 (19), also possesses an inhibitory phosphorylation site located close to the NLS (46, 47). Thus, the inhibition by BRAP2 of nuclear import of cellular proteins recognized by IMPα/β is

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**Figure 8.** BRAP2 can act as an NRNI for the tumor suppressor p53, but not hnRNPA1. A) CLSM images for COS-7 cells transfected to express GFP-BRAP2(2–592) followed by fixation 16 h post-transfection and immunostaining using specific anti-p53 (left panels) or anti-hnRNPA1 (right panels) antibodies together with Alexa568-coupled secondary antibody. B) Image analysis of CLSM images represented in A, performed as per Fig. 1: average $F_n$ (left), $F_c$ (middle), and $F_{n/c}$ ratio (right) ($n>45$); $F_n$ and $F_c$ values are expressed as percentages relative to p53 or hnRNPA1, respectively. $P$ values denote significant differences between presence vs. absence of GFP-BRAP2(2–592).
Figure 9. Proposed model of action of BRAP2 as an NRNI for specific viral proteins (A), dependent on phosphorylation near the NLS (B). A) Model of BRAP2 action. 1a) T-ag (T) NLS (red oval) mediates IMPα/β-mediated nuclear import (green arrows) (9, 15). 1b) On phosphorylation at Thr124 by Cdk (red arrow), BRAP2 is able to bind the T-ag NLS to prevent IMPα/β binding, and sequester T-ag in the cytoplasm (10). 2a: CMV ppUL44 (“44”) follows an IMPα/β-mediated NLS-dependent nuclear import pathway (green arrows) similar to that of T-ag (5). 2b) On phosphorylation at Thr427 by PK-C (red arrow), BRAP2 is able to bind the ppUL44 NLS to prevent IMPα/β binding, and sequester ppUL44 in the cytoplasm (3). CMV pUL30 (“30”) follows an IMPα/β-recognized NLS (red oval), which allows for nuclear import mediated by IMPα/β (green arrows) but does not contain a phosphorylation site adjacent/within the NLS (22) and hence is not recognized by BRAP2 and shows no inhibition of nuclear import. B) Phosphorylation-regulated NLSs (prNLSs) of T-ag and ppUL44 compared to similar sequences in the cellular factors p53 (44, 45) and p21Cip1 (19, 46, 47). Single-letter amino acid code is used, with basic amino acids of the NLS in bold; key phosphorylation sites are numbered according to their residue number in the protein.

likely to be phosphorylation regulated, in similar fashion to viral gene products.

DISCUSSION

This study documents for the first time the ability of the cytoplasmically localized protein BRAP2 to inhibit the nuclear import of the T-ag and ppUL44 proteins, but not HSV UL30 or HIV-1 Tat, dependent on phosphorylation near the NLS. Figure 9 summarizes the data here and elsewhere that indicate that BRAP2's NRNI activity is specific to IMPα/β-recognized proteins (Fig. 9B) that contain NLSs with flanking phosphorylation sites (phosphorylation-regulated NLSs or prNLSs) (2, 3, 6), making it a novel regulator of nuclear import. Intriguingly, BRAP2 appears to be an NRNI not only of specific viral proteins, but also of cellular proteins such as the p53 tumor suppressor and p21\(^{Cp1}\), apparently through similar phosphorylation-dependent mechanisms (6, 7, 14–17).

Exactly how the function of an NRNI such as BRAP2 may compete with/be integrated into nuclear import processes in the context of the whole cell is not clear at this stage, but since the relative binding affinities of IMPα/β and BRAP2 for NLS-containing proteins are in the low nanomolar range (18), the balance between import and cytoplasmic retention of IMPα/β-BRAP2 “targets” can be postulated firstly to be strongly dependent on their relative expression levels. Clearly, in the HL60 and analogous U937 cell systems (19), increased expression of BRAP2 in response to physiological stimuli results in reduced nuclear accumulation of endogenous proteins (p21\(^{Cp1}\); ref. 19), as well as ectopically expressed protein (T-ag; Fig. 7). Further, control over phosphorylation near the respective NLSs, on which BRAP2 binding is so dependent, enables fine tuning of the extent of cytoplasmic retention, thereby regulating precisely the levels and function of specific nuclear proteins; clearly, this is likely to be of great significance in virally infected cells in the case of key proteins involved in replication, such as T-ag and ppUL44, but also in the case of cellular proteins such as p53 and p21\(^{Cp1}\), which have important roles in growth regulation, differentiation, and transformation (19). Clearly, BRAP2’s role in this context is likely to have wide significance, since it is conserved through evolution, with close homologs in lower eukaryotes such as Drosophila melanogaster and Caenorrhabditis elegans (48).

In this context, note that although cytoplasmic retention of NLS-containing import cargoes was initially reported for NF-κB p65, which is retained in the cytoplasm by IκB (7, 14), and the glucocorticoid receptor, which interacts specifically with heat-shock protein HSP90 (17), the results here indicate that BRAP2 is quite distinct, in that it shows a rather broader specificity of IMPα/β-recognized NLS-containing target proteins, both viral and cellular, as well as exhibiting binding strongly dependent on phosphorylation adjacent to the NLSs. The dependence of BRAP2 binding/cytoplasmic retention on phosphorylation near the NLS (see also above) inevitably means that the effects of BRAP2 in preventing nuclear localization are strongly dependent on the signaling pathways triggering phosphorylation at the NLS-flanking phosphorylation sites. Notably, this explains why BRAP2’s role is not “all or none” in terms of keeping proteins out of the nucleus, but rather, crucial in conferring fine control over the nuclear concentration of key nuclear-acting proteins. That the extent of nuclear localization (rather than simple presence or absence in the nucleus) is a key driver of developmental processes/signal transduction has been shown for human sex determination and neuronal cell function with respect to the SRY/SOX9
and NF-κB p65 transcription factors, respectively (28, 49–52), and that it is critical in viral infection has been shown in studies for a range of viral pathogens, including cytomegalovirus (53), porcine reproductive and respiratory syndrome virus (54), respiratory syncytial virus (55), and Dengue (56). In the context of viral pathogenesis, analysis of BRAP2’s role in infected cells is a focus of future work in this laboratory; deeper understanding of BRAP2’s role as a NRNI for viral proteins will undoubtedly assist in the development of urgently needed approaches to combat viral disease.

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